

# PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

**PCT**

## NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Commissioner  
US Department of Commerce  
United States Patent and Trademark  
Office, PCT  
2011 South Clark Place Room  
CP2/5C24  
Arlington, VA 22202  
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

<b>Date of mailing (day/month/year)</b> 08 March 2001 (08.03.01)	
<b>International application No.</b> PCT/EP00/07108	<b>Applicant's or agent's file reference</b> WO 0452
<b>International filing date (day/month/year)</b> 25 July 2000 (25.07.00)	<b>Priority date (day/month/year)</b> 26 July 1999 (26.07.99)
<b>Applicant</b> LUCONI, Michaela et al	

1. The designated Office is hereby notified of its election made:

☒ in the demand filed with the International Preliminary Examining Authority on:

19 January 2001 (19.01.01)

☐ in a notice effecting later election filed with the International Bureau on:

2. The election ☒ was

☐ was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO  
34, chemin des Colombettes  
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer

C. Cupello

Telephone No.: (41-22) 338.83.38

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
1 February 2001 (01.02.2001)

PCT

(10) International Publication Number  
**WO 01/07021 A2**

- (51) International Patent Classification<sup>7</sup>: **A61K 31/00**
- (21) International Application Number: **PCT/EP00/07108**
- (22) International Filing Date: **25 July 2000 (25.07.2000)**
- (25) Filing Language: **English**
- (26) Publication Language: **English**
- (30) Priority Data:  
**FI99A000171** **26 July 1999 (26.07.1999)** **IT**
- (71) Applicant (*for all designated States except US*): **APPLIED RESEARCH SYSTEMS ARS HOLDING N.V.** [NL/NL]; Pietermaai 15, Curaçao (AN).
- (72) Inventors; and
- (75) Inventors/Applicants (*for US only*): **LUCONI, Michaela** [IT/IT]; Via del Campo di Marte, 9, I-50137 Firenze (IT). **BALDI, Elisabetta** [IT/IT]; Via Ussi, 54, I-50018 Scandicci-Firenze (IT). **FORTI, Gianni** [IT/IT]; Via Lambruschini, 4/a, I-50134 Firenze (IT).
- (54) Title: **PROCESS FOR THE IMPROVEMENT OF SPERMATOZOA FERTILIZATION ACTIVITY**
- (57) Abstract: The invention relates to a process for the improvement of spermatozoa fertilization activity, in particular for the increase of spermatozoa motility by inhibiting the enzyme phosphatidylinositol-3 kinase (PI3K). The invention further relates to uses and methods of PI3K inhibitors in infertility and assisted reproduction techniques (ART) as well as to a medium for storage and/or transportation of spermatozoa comprising PI3K inhibitors. The PI3K inhibitors are selected from the group consisting of 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002), wortmannin, quercetin, and derivatives and analogues thereof.
- (81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.
- (84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

**Published:**

— *Without international search report and to be republished upon receipt of that report.*

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*



**WO 01/07021 A2**

## PROCESS FOR THE IMPROVEMENT OF SPERMATOOZOA FERTILIZATION ACTIVITY

### FIELD OF THE INVENTION

The invention relates to a process for the improvement of spermatozoa fertilization activity, in particular for the increase of spermatozoa motility by inhibiting the enzyme phosphatidylinositol-3 kinase (PI3K). The invention further relates to uses and methods of PI3K inhibitors in infertility and assisted reproduction techniques as well as to a medium for storage and/or transportation of spermatozoa comprising PI3K inhibitors.

### BACKGROUND OF THE INVENTION

The infertility of a couple is defined as the inability of the woman to conceive after at least a year of regular unprotected sexual relations. Infertility may be caused by a multitude of factors, in which male factors play a fundamental role in around 40-50% of cases. Reduced male fertility is generally linked to alterations in seminal parameters such as morphology, motility and sperm count.

Various assisted fertilization techniques (ARTs) are proposed as treatment for infertility of the couple, in many cases making it possible to overcome the problem of both male and the female factors. These methods, the choice of which depends on the type of diagnosis made, may involve the collection of male and female gametes (spermatozoa and oocytes). The further treatment varies according to the cause of the infertility. The gametes may be transferred directly into the Fallopian tube (GIFT= gamete intra fallopian transfer) or are brought into contact with each other in a test tube. If the latter leads to fertilization of the oocyte, the resulting zygote or embryo is transferred into the uterus (IVFET = In Vitro Fertilization and Embryo Transfer).

When infertility is due to male factor(s), parameters of the seminal liquid and in particular the count and motility of spermatozoa determine the choice of the particular assisted fertilization method used. In the most serious cases of male-factor infertility the spermatozoa count and/or their motility is very low. The fertilization activity of semen is usually assessed in a spermogram. According to WHO standards, which can be taken from the "WHO manual" (WHO laboratory manual for the examination of human semen and sperm-cervical mucus interactions, 4<sup>th</sup> edition, Cambridge University Press 1999), semen are classified into the following groups:

- Normozoospermia: When all the spermatozoal parameters are normal together with normal seminal plasma, WBCs (White blood cells) and no agglutination;

- Oligozoospermia: When sperm concentration is  $< 20$  million/ml;
- Teratozoospermia: Fewer than 50% spermatozoa with forward progression(categories (a) and (b)) or fewer than 25% spermatozoa with category (a) movement;
- Asthenozoospermia: Fewer than 50% spermatozoa with normal morphology;
- Oligoasthenoteratozoospermia: Signifies disturbance of all the three variables (combination of only two prefixes may also be used);
- Azoospermia: No spermatozoa in the ejaculate.

Normal values of semen parameters have been issued by WHO that are generally used as reference. The fraction of motile sperm in semen is measured either by manual counting or using a computer assisted semen analysis (CASA) system. Motility is assessed at the time of semen liquefaction and after 1 and 3 hours to detect asthenozoospermia. Manual counting classifies sperm cells into 4 categories (immotile, locally motile, non linear and linear motile) using qualitative subjective criteria of selection. Many infertility centers now use CASA systems for objective measurements of sperm motion and positive correlations have been found between motion parameters such as the amplitude of lateral head displacement, curvilinear velocity, linearity and straight-line velocity and fertilization rates *in vitro* but the threshold levels for these motion characteristics have not yet been established to meet a general consensus.

In case of severe male factor infertility, micro-assisted fertilization techniques can be used. Among these techniques, intracytoplasmatic sperm injection (ICSI) is the most common and has the highest percentage of success. However, the safety of the ICSI procedure for the health of the resulting conceptus or embryo is still matter of debate (Edwards, 1999; Luetjens et al., 1999). In addition, ICSI is far more expensive and more time consuming as compared to IVF.

Thus, the possibility to recover a higher number of spermatozoa showing a higher motility could allow several oligoasthenospermic men to enter IVF rather than ICSI programs.

Various methods have attempted at increasing the motility of the spermatozoa, like treatment of spermatozoa with pentoxyphyllene, platelet activating factor or progesterone, for instance. However, the results obtained are variable and the responsiveness of the spermatozoa is not predictable.

Therefore, the finding of new methods and agents to improve sperm cell motility, leading to an improvement of the fertilization activity or fertilization rate, is highly desirable and urgently needed.

Phosphatidylinositol-3-kinases (PI3Ks) belong to a family of enzymes involved in signal transduction of tyrosine kinase receptors. Phosphatidylinositol-3-kinases, also called phosphoinositide-3-kinases (PI3Ks) generate lipids which are implicated in receptor-stimulated signalling and in the regulation of membrane traffic. Several distinct classes of PI3Ks have been identified that have been conserved throughout eukaryotic evolution. Potential signalling pathways downstream of PI3Ks have been elucidated and PI3K function is being characterized in several model organisms, as reviewed e.g. by Vanhaesebroeck et al. (1997). PI3Ks are heterodimeric enzymes present in various isoforms and composed of a catalytic subunit of 110 kDa, which is associated with a regulating subunit of 85 kDa.

In somatic cells phosphoinositide-3-kinases (PI3-kinases) are activated upon interaction with both receptor tyrosine kinases (RTK) and G-proteins resulting in the production of moieties involved in the inositol phospholipid signalling pathway. The enzyme is also present and active in human spermatozoa.

Several selective inhibitors of PI3Ks have been described. Wortmannin is one of the most well-known specific inhibitors. Wortmannin and analogs thereof are described, for example, in EP 0 635 268 A1, EP 0 648 492 A2 or EP 0 658 343 A1. These compounds are known to be involved in the treatment of neoplasms, atherosclerosis, and bone disorders. Other phosphatidylinositol-3-kinase inhibitors are 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002), described in Vlahos et al. (1994 and 1995), related to the bioflavonoid quercetin (Vlahos et al., 1994), for example.

None of these phosphatidylinositol-3-kinase inhibitors have been described to improve spermatozoa fertilization activity so far or are known to be involved in fertilization.

## SUMMARY OF THE INVENTION

It has now been found in accordance with the invention that inhibitors of the enzyme phosphatidylinositol-3-kinase can significantly improve the parameters determining sperm cell fertilization activity, in particular the sperm cell motility.

The invention therefore relates to a process for enhancing spermatozoa fertilization activity, in particular for increasing the motility of the spermatozoa, comprising treating the spermatozoa with a phosphatidylinositol-3 kinase (PI3K) inhibitor. The invention further relates to spermatozoa in which the activity of the phosphatidylinositol-3 kinase is inhibited, as well as to the use of a PI3K inhibitor for improving the fertilization rate in assisted reproduction techniques (ART). A third aspect of the invention concerns the use of a phosphatidylinositol-3 kinase (PI3K) inhibitor for the manufacture of a medicament for the treatment of infertility, in particular male infertility. A fourth aspect of the present invention relates to methods of ART therapy comprising treating spermatozoa with an phosphatidylinositol-3-kinase inhibitor. A fifth aspect of the invention relates to a medium for storage and/or transportation of spermatozoa comprising a phosphatidylinositol-3-kinase inhibitor.

## BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the effect of 10 $\mu$ M of the PI3K inhibitor LY294002 on the motility of spermatozoa.

Fig. 1 A: Rapid progressive component in three individual patients. Each symbol represents one individual patient. Fig. 1 B: Rapid progressive motility (a) and rapid + slow progressive motility (a+b) of the spermatozoa; mean values  $\pm$  SEM. P indicates the p-value. LY indicates LY294002.

Fig. 2 shows the effect of 100 $\mu$ M LY294002 on the progressive motility of the spermatozoa obtained by the swim-up method.

Fig. 3 shows the effect of 10 $\mu$ M and 100 $\mu$ M LY294002 on the progressive motility of spermatozoa obtained by the swim-up method; mean values  $\pm$  SEM. C indicates control, LY indicates LY294002. The insert shows the effects of 100 $\mu$ M LY294002 on the vitality of spermatozoa obtained by the swim-up method.

Fig. 4 shows the effect of 10 $\mu$ M LY294002 on the progressive motility of spermatozoa obtained by the swim-up method. Fig. 4 A shows the effect on the rapid progressive component (motility a) in individual patients. Each symbol indicates an individual patient. Fig. 4 B shows a histogram depicting the effect on the fast progressive motility (a) and the fast plus slow progressive motility (a+b) of spermatozoa (mean values  $\pm$  SEM).

Fig. 5 shows a dose-response curve of increasing concentrations of LY294002 ( $\mu\text{M}$ ) (x-axis) on the rapid forward motility of the spermatozoa (y-axis) obtained by the swim-up method.

Fig. 6 shows a histogram depicting the sperm parameters VCL (percentage curvilinear velocity), VAP (average path velocity), VSL (straight-line velocity) and HA (hyperactivated sperm fraction), as determined by computer aided sperm analysis (CASA) in sperm samples from 12 different oligoasthenospermic subjects.

Fig. 7 shows a histogram depicting the effect of different amounts of Lithiumchloride (LiCl) with and without 10  $\mu\text{M}$  of LY294002 on the percentage forward motility of sperm cells.

### DESCRIPTION OF THE INVENTION

It is an object of the present invention to provide a process for improving the spermatozoa fertilization activity, in particular for increasing the motility of spermatozoa. The process according to the invention comprises treating the spermatozoa with a phosphatidylinositol-3 kinase (PI3K) inhibitor.

The invention is based on the finding that phosphatidylinositol-3-kinase inhibitors have a pronounced positive effect on parameters determining sperm cell fertilization activity, i.e. the parameters relevant to the capacity of sperm cells to fertilize an oocyte. The most important factors involved in the ability to fertilize are the number of active sperms and the motility of the spermatozoa. According to the WHO manual, motility of 50% is considered the lower limit of normality.

It has now been found in accordance with the invention that the number of motile sperms obtainable from semen samples as well as the motility of the individual spermatozoa can be significantly increased by PI3K inhibitors, thus indicating the role of PI3K inhibitors as enhancers of fertility. This effect is detectable in normospermic individuals. However, it is even more marked in spermatozoa displaying pathogenic features, like oligoasthenospermic patients, i.e. those patients having a reduced total number of spermatozoa and a reduced spermatozoa motility. The invention renders it possible to increase the percentage of spermatozoa with progressive motility, thus significantly improving the probability of successful fertilization. Thus, the process according to the invention helps patients avoid using ICSI in favor of less invasive ART, like conventional IVF.

The terms "spermatozoa" or "sperm (cells)" are used synonymously herein and relate to male gametes. "Semen" or "seminal fluid/liquid" contain sperm cells as well as seminal plasma.

The term "increase of spermatozoa fertilization activity" means any enhancement, improvement, or change to the better of the parameters determining the quality or activity of the sperm cell, such as e.g. percentage curvilinear velocity (VCL), average path velocity (VAP), straight-line velocity (VSL) and hyperactivated sperm fraction (HA). The quality of the spermatozoa determines the fertilization rate in assisted reproduction techniques.

The term "increase" of spermatozoa motility as used herein is meant to encompass any improvement, enhancement, amelioration or change to the better of the quality or fertilization activity or motility or velocity of the cells.

The term "Phosphatidylinositol-3-kinase" or "PI3K" as used herein encompasses any member of the PI3K family, i.e. those related enzymes having the activity outlined in the introduction.

An "inhibitor of phosphatidylinositol-3-kinase" is also referred to as PI3K and inhibits the production of D-3 phosphoinositides in the cell. The term D-3 phosphoinositides is intended to encompass derivatives of phosphatidylinositol that are phosphorylated in the D-3 position of the inositol ring and comprises, for example, phosphatidylinositol(3)monophosphate (PI(3)P), phosphatidylinositol(3,4)bispophosphate (PI(3,4)P<sub>2</sub>) or phosphatidylinositol(3,4,5)trisphosphate (PI(3,4,5)P<sub>3</sub>).

In a preferred embodiment, treating the spermatozoa with a phosphatidylinositol-3 kinase (PI3K) inhibitor is performed on the seminal liquid comprising the spermatozoa. Performing the method according to the invention directly on the seminal liquid without any further treatment has the advantage that it is simple and fast. Since the PI3K inhibitor of the invention enhances sperm cell motility, removal of the seminal plasma is not necessary.

In a further preferred embodiment, the process further comprises separating the spermatozoa by spermatozoa separation methods used in assisted reproduction techniques (ART).

Since seminal plasma contains factors that inhibit capacitation and fertilization as well as a considerable amount of non-motile spermatozoa even in a fertile individual, it is advantageous to separate motile sperm cells from fluid, non-motile and morphologically defective spermatozoa. This step is essential in traditional ART like IVF, GIFT or IUI. It leads to an enhancement of the



fertilization success rate also in the process according to the invention. It is evident from the examples that the increase in spermatozoa motility by a phosphatidylinositol-3-kinase inhibitor is even more pronounced in spermatozoa which have been separated from the seminal plasma.

In a further preferred embodiment of the invention, separating the spermatozoa is performed by a method selected from the wash and spin method, the sedimentation method, the direct swim-up method, the pellet and swim-up method, and the buoyant density gradient method.

These methods are well known in the art. They are traditionally used in assisted reproduction techniques and described in detail in "A textbook of In vitro Fertilization and Assisted Reproduction, The Bourn Hall guide to clinical and laboratory practice, editor: Peter R. Brinsden, The Parthenon Publishing Group" (1999) on pages 204 to 208. This textbook is referred to hereinafter as the "Bourn Hall guide".

Preferably, separating the spermatozoa is performed by the direct swim-up method.

This method implies self-selection of motile sperms, essentially comprising layering an aliquot of medium on top of a semen sample and allowing it to stand a room temperature for a certain period of time. The motile sperm cells will migrate into the top layer (medium), from which they can be recovered. The method may also include centrifugation step(s).

The advantage of "swim-up" selected spermatozoa is that the motile cells present in the sample are isolated and concentrated and that the proportion of morphologically normal sperm is increased. It is shown in the examples that the process according to the invention leads to an increase the amount of spermatozoa recovered from seminal fluid by the swim-up method. This is due to the increased motility of the sperms, which therefore migrate more quickly and in higher amounts into the upper phase of the sample.

The method may be varied and combined with further isolation/separation techniques, depending on the amount of motile cells in the sample. For example, the swim-up procedure may be performed through the layering of 1 ml of medium containing albumin on a 1 ml of underlying seminal liquid in a test tube. After one hour of incubation at 37°C in the air or in 5% CO<sub>2</sub> the upper phase of the medium to which the spermatozoa with better motility characteristics have migrated is collected. This technique may also comprise or be combined with a centrifugation step, for example

centrifugation on Percoll gradients. The separated, isolated or enriched spermatozoa are then used in assisted-reproduction techniques or may be deep-frozen before being further processed, for example.

Advantageously, the incubation of spermatozoa with the PI3K inhibitor is carried out on the seminal fluid, and then swim-up selection is performed. Thereafter, the spermatozoa may be washed one or several times to eliminate the PI3K inhibitor, before being further processed for fertilization.

Preferably, the process according to the invention is performed on mammal spermatozoa, in particular on human spermatozoa.

In a highly preferred embodiment, the PI3K inhibitor is selected from the group consisting of 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002), wortmannin, quercetin, and derivatives and analogues thereof.

LY294002 is the chemical compound 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one, as described by Vlahos et al. (1994). Derivatives or analogues thereof may also be used according to the invention.

Wortmannin is a fungal metabolite derived from *T. wortmannii* (Kyowa Hakko Kohyo Co. Ltd.) or from *P. fomiculosum* (Sigma). Wortmannin and its derivatives are described in the European Patent Applications EP 0 635 268 A1, EP 0 648 492 A2 or EP 0 658 343 A1, for example.

A further phosphatidylinositol-3-kinase inhibitor which can be used according to the invention is the bioflavonoid quercetin, or derivatives or analogues thereof. Vlahos et al. (1994) discloses examples of quercetin and its derivatives or analogues.

A process wherein the PI3K inhibitor is LY 294002 is highly preferred according to the invention. This agent has been shown to be particularly efficacious for the enhancement of sperm fertilization activity.

Preferably, the spermatozoa are treated with an amount of PI3K inhibitor in the range of about 0.01 to 1000  $\mu$ M, more preferably of about 5 to 500  $\mu$ M and most preferably of about 10 to 100  $\mu$ M. Treating the spermatozoa with the PI3K inhibitor advantageously comprises incubating the

spermatozoa for a period of time in the range of about 30 minutes to 10 hours, preferably about 1 to 8 hours, most preferably about 2 to 6 hours at a temperature of about 37°C.

It is a further object of the invention to provide spermatozoa having an improved ability of fertilization. Therefore the invention further relates to spermatozoa in which the activity of the phosphatidylinositol-3 kinase is inhibited. The invention also relates to spermatozoa obtainable by the process described above. The spermatozoa in which the phosphatidylinositol-3-kinase is inhibited or which were obtained in a process according to the invention exhibit an improved fertilization activity, a higher motility as compared to untreated sperm cells and thus exhibit a better performance with regard to fertilization.

As mentioned above, sperm cell fertilization activity determines the fertilization rate in ART. The invention therefore further relates to the use of a PI3K inhibitor for improving the fertilization rate in assisted reproduction techniques (ART).

Any assisted reproduction method known in the art may be used according to the invention. In preferred embodiments, the assisted reproduction techniques are selected from in vitro fertilization (IVF), gamete intrafallopian transfer (GIFT), and intrauterine insemination (IUI).

The invention further relates to the use of a phosphatidylinositol-3 kinase (PI3K) inhibitor for the manufacture of a medicament for the treatment of infertility, in particular male infertility. While the invention is described in more detail for in vitro fertilization techniques, it will be appreciated by the person skilled in the art that the compound may be as efficient in terms of activity when administered *in vivo*.

In this case, the medicament is preferably presented in the form of a pharmaceutical composition comprising the phosphatidylinositol-3-kinase inhibitor together with one or more pharmaceutically acceptable carriers and/or excipients. Such pharmaceutical compositions form yet a further aspect of the present invention.

An "effective amount" refers to an amount of the active ingredients that is sufficient to affect the fertilization activity, in particular the mobility of spermatozoa. The effective amount will depend on the route of administration and the condition of the patient.

"Pharmaceutically acceptable" is meant to encompass any carrier, which does not interfere with the effectiveness of the biological activity of the active ingredient and that is not toxic to the host to which is administered. For example, for parenteral administration, the above active ingredients may be formulated in unit dosage form for injection in vehicles such as saline, dextrose solution, serum albumin and Ringer's solution. Besides the pharmaceutically acceptable carrier, the compositions of the invention can also comprise minor amounts of common additives, such as stabilisers, excipients, buffers and preservatives.

The administration of such active ingredient may be by intravenous, intramuscular or subcutaneous route. Other routes of administration, which may establish the desired blood levels of the respective ingredients, are comprised by the present invention.

The invention further relates to the use of a phosphatidylinositol-3 kinase (PI3K) inhibitor for the manufacture of a medicament for the improvement of spermatozoa fertilization activity, in particular for the increase of spermatozoa motility.

In preferred embodiments, the PI3K inhibitor is selected from the group consisting of 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002), wortmannin, quercetin, and derivatives and analogues thereof. The use of LY294002 as phosphatidylinositol-3-kinase inhibitor is highly preferred.

It is a further object of the present invention to provide for an improvement of ART therapy. The improvement consists in including into known techniques for assisted fertilization a step comprising treating spermatozoa with a phosphatidylinositol-3 kinase (PI3K) inhibitor. The further steps used in assisted reproduction techniques are well known to the person skilled in the art and can be taken from the WHO manual (supra) or the Bourn Hall guide (supra).

In a preferred embodiment of the invention, the ART are selected from in vitro fertilization (IVF), gamete intrafallopian transfer (GIFT), or intrauterine insemination (IUI).

It is a further object of the present invention to provide a medium for storage and/or transportation of spermatozoa having improved qualities. The invention therefore also relates to a medium comprising a phosphatidylinositol-3 kinase (PI3K) inhibitor. Apart from the PI3K inhibitor, the medium may contain any further component known to be useful for storage and/or transportation,

depending on the kind of storage and/or transportation required. For example, the spermatozoa may be stored at room temperature or by cryopreservation. The latter is common for the storage of the cells for a longer period of time. Specific examples of further components of the medium can be taken e.g. from WO 97/16965. Further specific media suitable for cryopreservation of semen are included in Appendix II, pp. 541 and 542 of the Bourn Hall guide (supra), for instance. They could be supplemented with a PI3K inhibitor according to the invention to improve the fertilization activity, in particular the motility of the sperms before fertilization takes place.

In a preferred embodiment, the medium comprises mammal spermatozoa, in particular human spermatozoa. Preferable, PI3K inhibitor present in the medium according to the invention is selected from the group consisting of 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002), wortmannin, quercetin, and derivatives and analogues thereof. In a highly preferred embodiment, PI3K inhibitor is LY294002.

In yet a further preferred embodiment, the medium according to the invention comprises amounts of the PI3K inhibitor in the range of about 0.01 to 1000  $\mu\text{M}$ , preferably of about 5 to 500  $\mu\text{M}$ , and most preferably of about 10 to 100  $\mu\text{M}$ .

Having now described the invention, it will be more readily understood through reference to the following examples that are provided by way of illustration and are not intended to be limiting the present invention.

### EXAMPLES

In the experiments the following examples are based on, standard methods of in vitro fertilization have been used. With regard to the details of these methods, reference is made to the "WHO manual" (WHO laboratory manual for the examination of human semen and sperm-cervical mucus interactions, 4<sup>th</sup> edition, Cambridge University Press 1999). In particular, the direct swim-up method can be taken from pp. 104 to 106 of this manual.

#### Example 1: Effect of LY294002 on the rapid motility of spermatozoa

Spermatozoa were prepared according to the standard procedures of IVF. Briefly, spermatozoa were prepared from 3 oligoasthenospermic subjects undergoing semen analysis for couple infertility after

informed consent. 10  $\mu$ M of the PI3K inhibitor LY294002 were added directly to the seminal liquid and incubated for 2 hours for two hours at 37°C and 5% CO<sub>2</sub>. The motility of the spermatozoa was then blindly evaluated under the microscope according to WHO manual procedures. The results of this evaluation are shown in Fig. 1. Incubation with the compound causes an increase of rapid motility of the spermatozoa in the seminal liquid (Fig. 1A). Fig. 1 B shows that the increase was observed not only in the rapid mobility (a), but also in the percentage of forward motility (rapid and slow component a + b).

In a group of 7 samples taken from seven individuals, the phosphatidylinositol-3-kinase inhibitor LY294002 was added in a higher concentration (100  $\mu$ M). After the incubation with the compound for two hours, swim-up selection of the spermatozoa was performed according to procedures described in the WHO-manual. The incubation of the sperm cells with a ten times higher concentration of the inhibitor (100  $\mu$ M) in combination with the swim-up selection resulted in a significant increase of progressive motility in all of the seven samples, as shown in Fig. 2.

Table I summarizes the results obtained in a similar experiment in 20 oligoasthenospermic patients. The compound was added in a concentration of 10  $\mu$ M. The effects of LY294002 addition to seminal plasma on the percentage of sperm forward motility both in seminal plasma and in the recovered swim-up fraction measured after incubation with the compound resulted in an increase of 39% of sperm forward motility in the seminal plasma and of 26% in swim-up selected sperm cells.

Table II summarizes the total sperm cell recovery in the swim-up, which was increased by 43% as compared to the control. Thus, incubation of semen with the phosphatidylinositol-3-kinase inhibitor leads to a significant increase in the recovery of spermatozoa during swim-up selection.

TABLE I

% Sperm Forward Motility (n=20)			
Sperm in Seminal Plasma		Swim up-selected sperm	
C	LY 10 $\mu$ M	C	LY 10 $\mu$ M
35.7 $\pm$ 5.1	49.8 $\pm$ 4.7 * <i>p</i> <0.001 ↑ 39%	54.2 $\pm$ 4.5	68.6 $\pm$ 3.9 * <i>p</i> <0.001 ↑ 26%

TABLE II

Sperm recovery in swim up ( $10^6$ )	
C	LY 10 $\mu$ M
13.3 $\pm$ 3.9 (n=20)	19.0 $\pm$ 4.8 * p<0.005 (n=20) ↑ 43%

Fig. 3 summarizes the results obtained in a similar experiment on samples from higher numbers of patients. The sperm cells were submitted to the swim-up selection method. Treatment with 10  $\mu$ M of the phosphatidylinositol-3-kinase inhibitor LY294002 resulted in an increase of 58% in the progressive motility (21 patients) as compared to the control (40 patients, without LY294002). Treatment of samples from 29 patients with 100  $\mu$ M of the inhibitor resulted in an increase of the motility of 67% as compared to the control, i.e. in an additional enhancement of spermatozoa motility of about 10%.

The effect of 100  $\mu$ M of LY294002 on the viability of the spermatozoa was also assessed (see insert of Fig. 3). The incubation for two hours with the LY294002 did not alter the vitality of the cells, as evaluated using the eosin test and hypo-osmotic swelling. Even after 48 hours of incubation, no effect on sperm viability was observed in any of the tested samples (not shown).

Fig. 4 shows the results of a further experiment carried out in the same manner as outlined above on samples from 12 individual patients. An improvement of percentage progressive motility after 2 hours of incubation of the sperm cells with LY294002 was clearly detectable, both on the rapid component (a) and on the rapid and slow component (a+b).

The experiments described above were performed on samples from oligoasthenospermic individuals. The effects on the stimulation of progressive motility by LY294002 are more pronounced the more serious the sperm pathology of patients is (oligospermia, asthenospermia, teratospermia). However, the effect of this compound could also be observed in normospermic subjects. The effect of incubation with 10  $\mu$ M LY 294002 for two hours followed by swim-up

selection resulted in an increase from  $53.5 \pm 2.0 \%$  to  $76.3 \pm 1.5 \%$  in the forward motility of spermatozoa from 88 randomly selected subjects (not shown).

This effect of LY294002 on samples from normospermic individuals was dose-responsive, as shown in Fig. 5. The values for the rapid forward motility increased with increased doses of the inhibitor applied (from 0 to 1000  $\mu\text{M}$ ). The values were calculated with respect to the control (zero) value. The expected sigmoidal curve demonstrates the dose-response effect of the inhibitor on the rapid forward motility of the treated versus untreated spermatozoa, showing an  $\text{EC}_{50}$  (effect dose of 50%) of about 1  $\mu\text{M}$ .

Similar effects on spermatozoa have been observed using an unrelated phosphatidylinositol-3-kinase inhibitor, wortmannin.

The experimental evidence described above demonstrates that two different inhibitors of the enzyme phosphatidylinositol-3-kinase are highly efficient in improving spermatozoa motility. This effect is particularly pronounced in pathogenic spermatozoa, but also detectable in normospermic individuals. The results presented above show that incubation of spermatozoa with an phosphatidylinositol-3-kinase inhibitor, if included in traditional assisted reproduction techniques, leads to an increase of sperm cell motility, thus enhancing the probability of fertilization in ART.

#### Example 2: Effect of LY294002 on further sperm cell parameters

The increase in forward motility, demonstrated in Example 1, was associated with an increase in sperm parameters related to fertilization activity of the spermatozoa *in vitro*, such as percentage curvilinear velocity (VCL), average path velocity (VAP), straight-line velocity (VSL) and hyperactivated sperm fraction (HA). These parameters were determined by computer aided sperm analysis (CASA) in sperm samples from 12 different oligoasthenospermic subjects. The results are shown in Fig. 6. Each of these parameters were increased in a statistically significant manner by incubation with 10  $\mu\text{M}$  of LY294002 as compared to the control sample, indicating a significant overall improvement of sperm cell fertilization activity.

Similar effects on spermatozoa have been observed using an unrelated phosphatidylinositol-3-kinase inhibitor, wortmannin.



These results demonstrate the overall improvement of the relevant parameters related to sperm cell fertilization activity by phosphatidylinositol-3-kinase inhibitors. Since all of the above parameters are essential for the fertilization rate obtained in assisted fertilization techniques, the invention provides for an agent useful for the treatment of severely impaired male-factor infertility.

**Example 3:** Effect of LY294002 on forward motility of H<sub>2</sub>O<sub>2</sub> or LiCl treated spermatozoa

It is well known that reactive oxygen species (ROS), which may be generated during sperm preparation for IVF, exert detrimental effects on sperm fertilization potential. In particular, among ROS, H<sub>2</sub>O<sub>2</sub> strongly reduces motility when added to sperm samples at micromolar concentrations. Therefore, the effect of LY294002 on H<sub>2</sub>O<sub>2</sub> treated sperm cells was evaluated. The compound was added to swim-up selected spermatozoa samples from oligoasthenospermic patients in amounts of 10  $\mu$ M either alone or in combination with 200  $\mu$ M of H<sub>2</sub>O<sub>2</sub>. It was observed that the detrimental effects exerted by 200  $\mu$ M H<sub>2</sub>O<sub>2</sub> on sperm motility were completely reversed by the addition of LY294002 to the incubation medium (Table III), demonstrating a protective activity of the compound with regard to ROS effects.

TABLE III

Control (%)	LY294002 (%)	H <sub>2</sub> O <sub>2</sub> (%)	LY294002+H <sub>2</sub> O <sub>2</sub> (%)
45.4 $\pm$ 5.0 (n=12)	71.7 $\pm$ 3.2** (n=12)	26.5 $\pm$ 4.9** (n=12)	52.6 $\pm$ 5.5* (n=12)

\*P<0.002 vs H<sub>2</sub>O<sub>2</sub>; \*\*P<0.0001 vs Control

LiCl is also a known inhibitor of sperm cell motility. An incubation of swim-up selected spermatozoa with 10  $\mu$ M LY294002 for two hours either with or without different concentrations of LiCl resulted in reversing the effect of LiCl induced inhibition of sperm motility (Fig. 7)

Similar effects on spermatozoa have been observed using an unrelated phosphatidylinositol-3-kinase inhibitor, wortmannin.

In this example, the activity of phosphatidylinositol-3-kinase inhibitors to rescue spermatozoa from deleterious agents, which may be generated in assisted fertilization techniques has been demonstrated. Therefore, the invention provides for a major improvement of ART, leading to a higher fertilization rate and eliminating some of the most serious drawbacks of these techniques.

## REFERENCES

- Edwards RG "Widening perspectives of intracytoplasmic sperm injection" (1999). *Nature Medicine* 5, 377-378.
- Luetjens CM, Payne C, Schatten G "Non-random chromosome positioning in human sperm and sex chromosome anomalies intracytoplasmic sperm injection (1999). *The Lancet* 353, 1240.
- Vanhaesebroeck B, Leervers SJ, Panayotou G, Waterfield MD "Phosphatidylinositol-3-kinases: a conserved family of signal transducers" (1997). *Trends. Biochem. Sci.* 22, 267-72.
- Vlahos CJ, Matter WF, Brown RF, Traynor Kaplan AE, Heyworth PG, Prossnitz ER "Investigation of neutrophil signal transduction using a specific inhibitor of phosphatidylinositol-3-kinase" (1995). *J. Immunol.* 154, 2413-22.
- Vlahos CJ, Matter WF, Hui KY, Brown RF "A specific inhibitor of phosphatidylinositol-3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002)" (1994). *J. Biol. Chem.* 269, 5241-48.

## CLAIMS

1. Process for improving the fertilization activity of spermatozoa, in particular for increasing spermatozoa motility, comprising treating the spermatozoa with a phosphatidylinositol-3 kinase (PI3K) inhibitor.
2. Process according to claim 1, wherein treating the spermatozoa with a phosphatidylinositol-3 kinase (PI3K) inhibitor is performed on seminal liquid comprising the spermatozoa.
3. Process according to claim 1 or 2, further comprising separating the spermatozoa by spermatozoa separation methods used in assisted reproduction techniques (ART).
4. Process according to claim 3, wherein separating the spermatozoa is performed by a method selected from the wash and spin method, the sedimentation method, the direct swim-up method, the pellet and swim-up method, the buoyant density gradient method.
5. Process according to claim 4, wherein separating the spermatozoa is performed by the direct swim-up method.
6. Process according to any of claims 1 to 5, wherein the process is performed on mammal spermatozoa, in particular on human spermatozoa.
7. Process according to any of claims 1 to 6, wherein the PI3K inhibitor is selected from the group consisting of 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002), wortmannin, quercetin, and derivatives and analogues thereof.
8. Process according to claim 7, wherein the PI3K inhibitor is LY294002.
9. Process according to any of claims 1 to 8, wherein spermatozoa are treated with an amount of PI3K inhibitor in the range of about 0.01 to 1000  $\mu\text{M}$ , about 5 to 500  $\mu\text{M}$ , or about 10 to 100  $\mu\text{M}$ .
10. Process according to any of claims 1 to 9, wherein treating the spermatozoa with the PI3K inhibitor comprises incubating the spermatozoa for a period of time in the range of about 30 minutes to 10 hours or about 1 to 8 hours or about 2 to 6 hours at a temperature of around 37°C.

11. Spermatozoa in which the activity of the phosphatidylinositol-3 kinase is inhibited.
12. Spermatozoa obtainable by the process according to any of claims 1 to 10.
13. Use of a PI3K inhibitor for improving the fertilization rate in assisted reproduction techniques (ART).
14. Use according to claim 12, wherein the assisted reproduction techniques are selected from in vitro fertilization (IVF), gamete intrafallopian transfer (GIFT), or intrauterine insemination (IUI).
15. Use of a phosphatidylinositol-3 kinase (PI3K) inhibitor for the manufacture of a medicament for the treatment of infertility, in particular male infertility.
16. Use of a phosphatidylinositol-3 kinase (PI3K) inhibitor for the manufacture of a medicament for improving spermatozoa fertilization activity, in particular for increasing spermatozoa motility.
17. Use according to any of claims 13 to 16, wherein PI3K inhibitor is selected from the group consisting of 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002), wortmannin, quercetin, and derivatives and analogues thereof.
18. Use according to claim 16, wherein the PI3K inhibitor is LY294002.
19. Method of ART therapy, comprising treating spermatozoa with a phosphatidylinositol-3 kinase (PI3K) inhibitor.
20. Method according to claim 19, wherein the ART are selected from in vitro fertilization (IVF), gamete intrafallopian transfer (GIFT), or intrauterine insemination (IUI).
21. A medium for storage and/or transportation of spermatozoa comprising a phosphatidylinositol-3 kinase (PI3K) inhibitor.

22. Medium according to claim 21 for the storage and/or transportation of mammal spermatozoa, in particular human spermatozoa.
23. Medium according to claims 21 or 22, wherein the PI3K inhibitor is selected from the group consisting of 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002), wortmannin, quercetin, and derivatives and analogues thereof.
24. Medium according to claim 23, wherein the PI3K inhibitor is LY294002.
25. Medium according to any of claims 21 to 24, comprising an amount of PI3K inhibitor in the range of about 0.01 to 1000  $\mu$ M, about 5 to 500  $\mu$ M, or about 10 to 100  $\mu$ M.

1/7

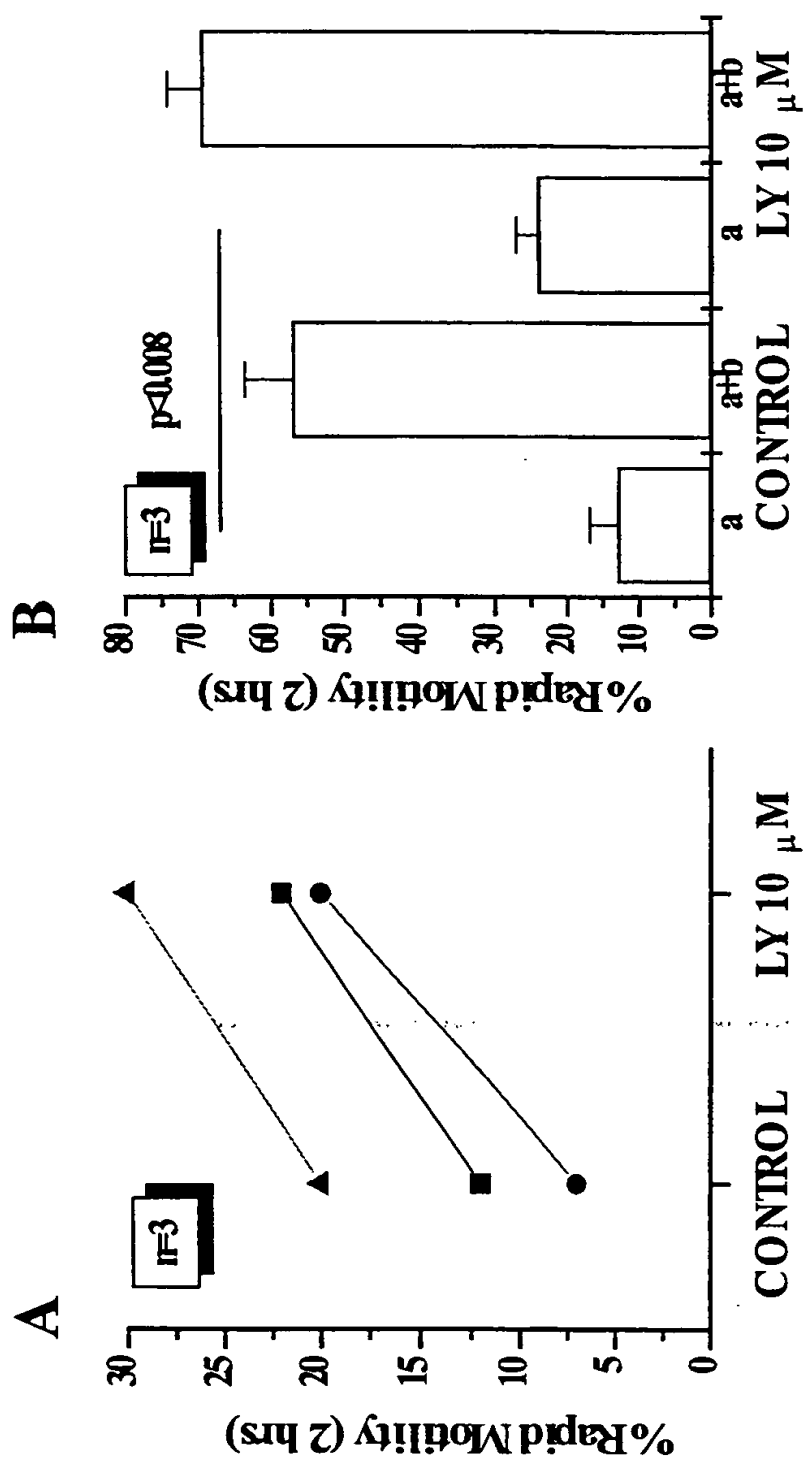


Fig. 1

2/7

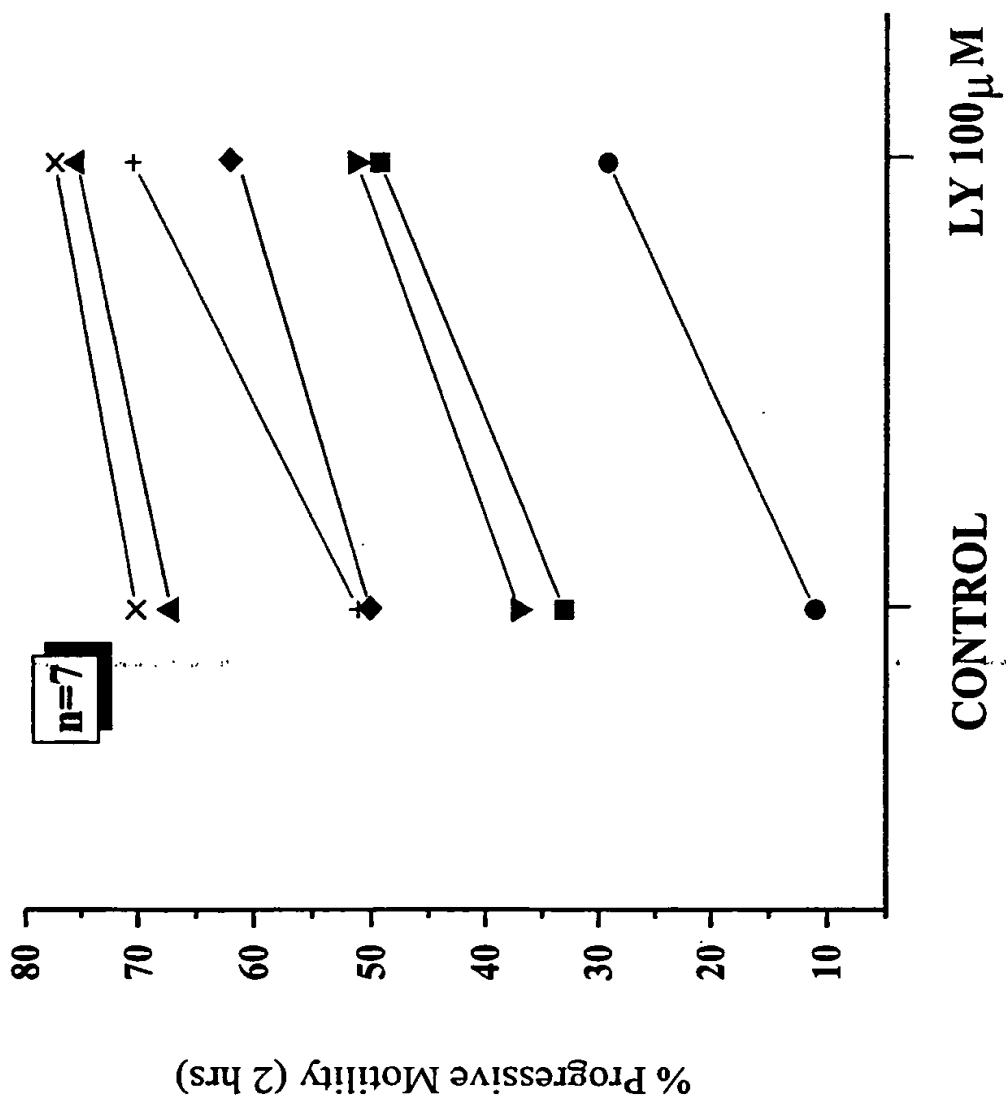


Fig. 2

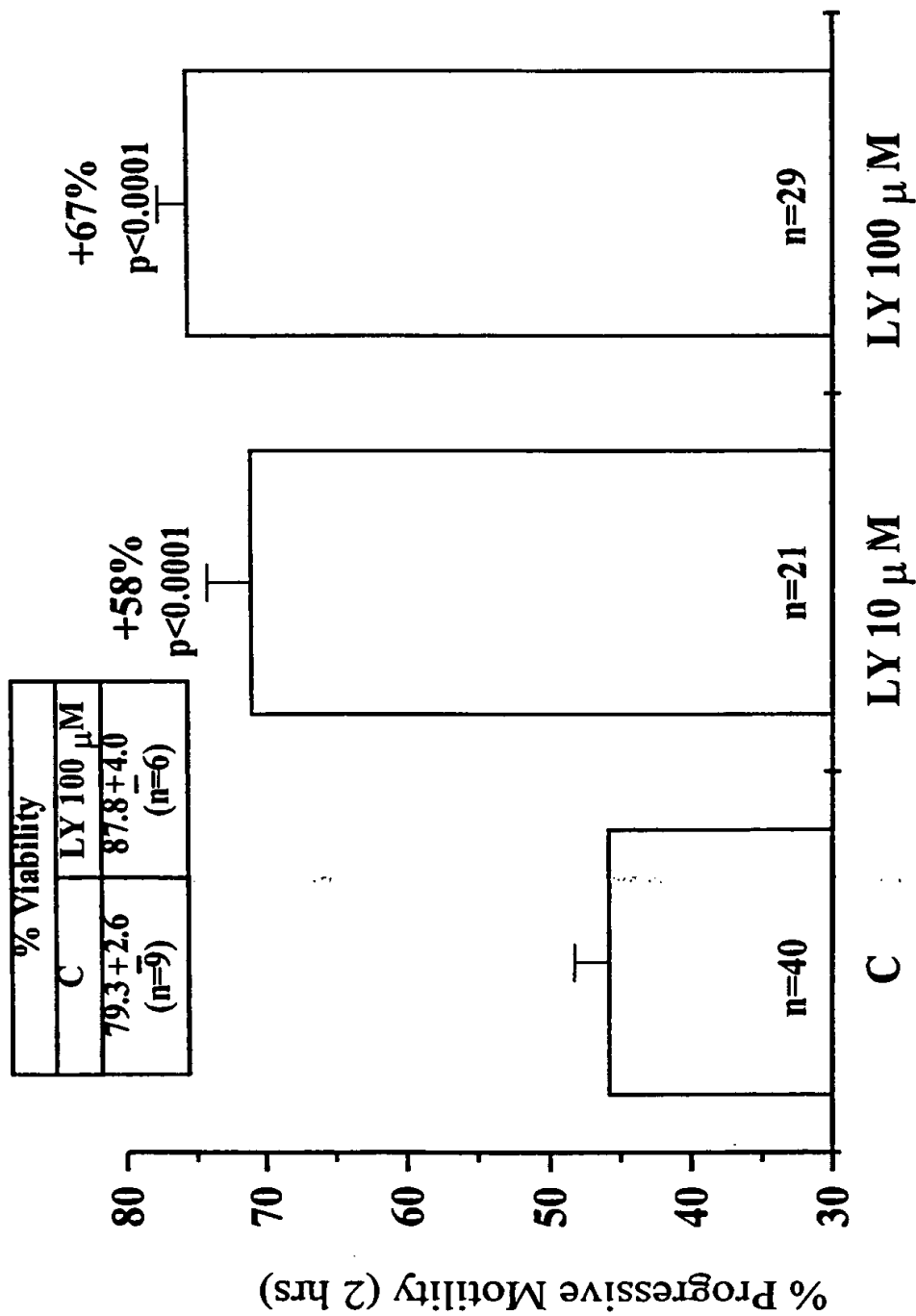


Fig. 3



4/7

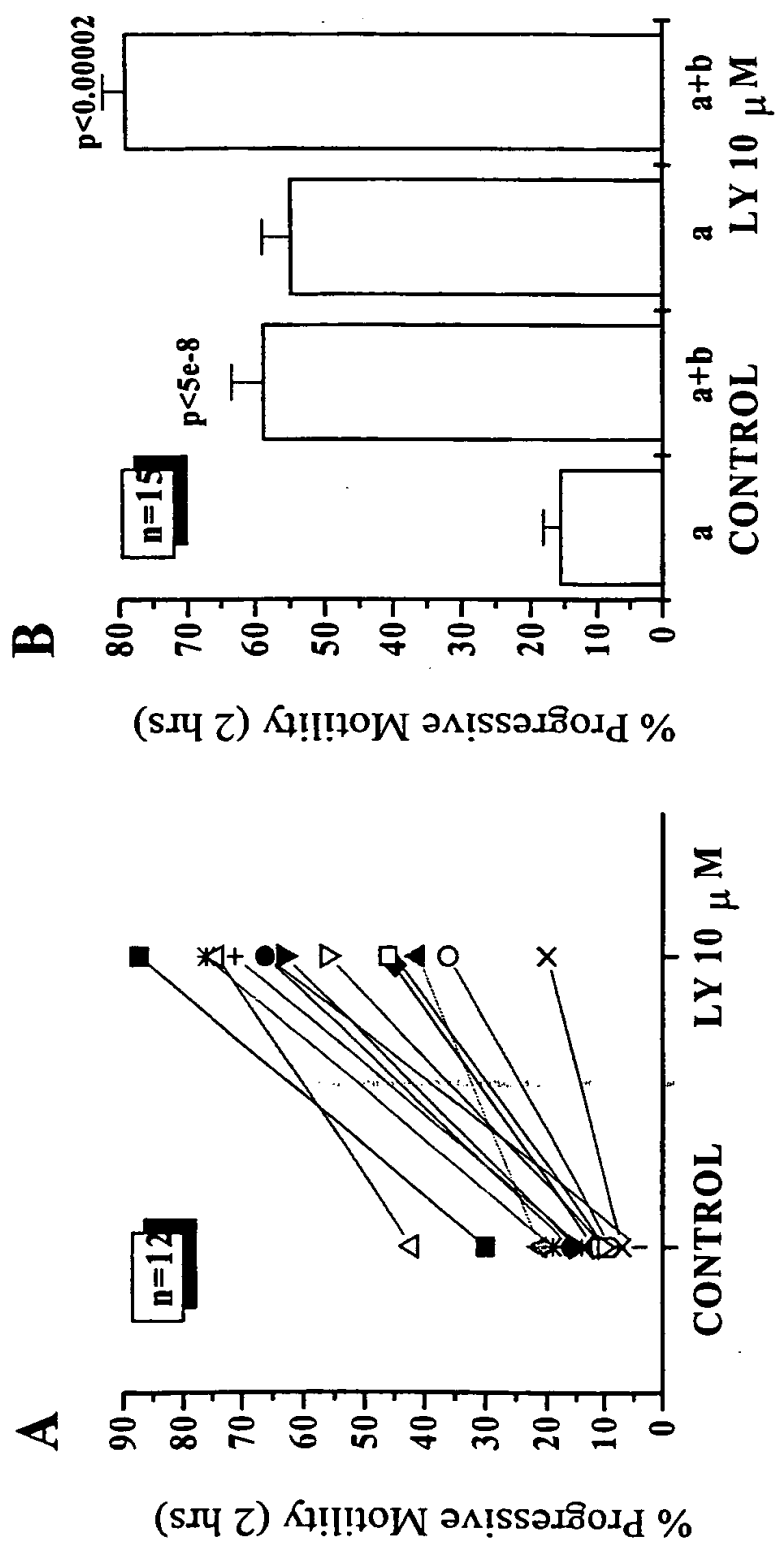


Fig. 4

5/7

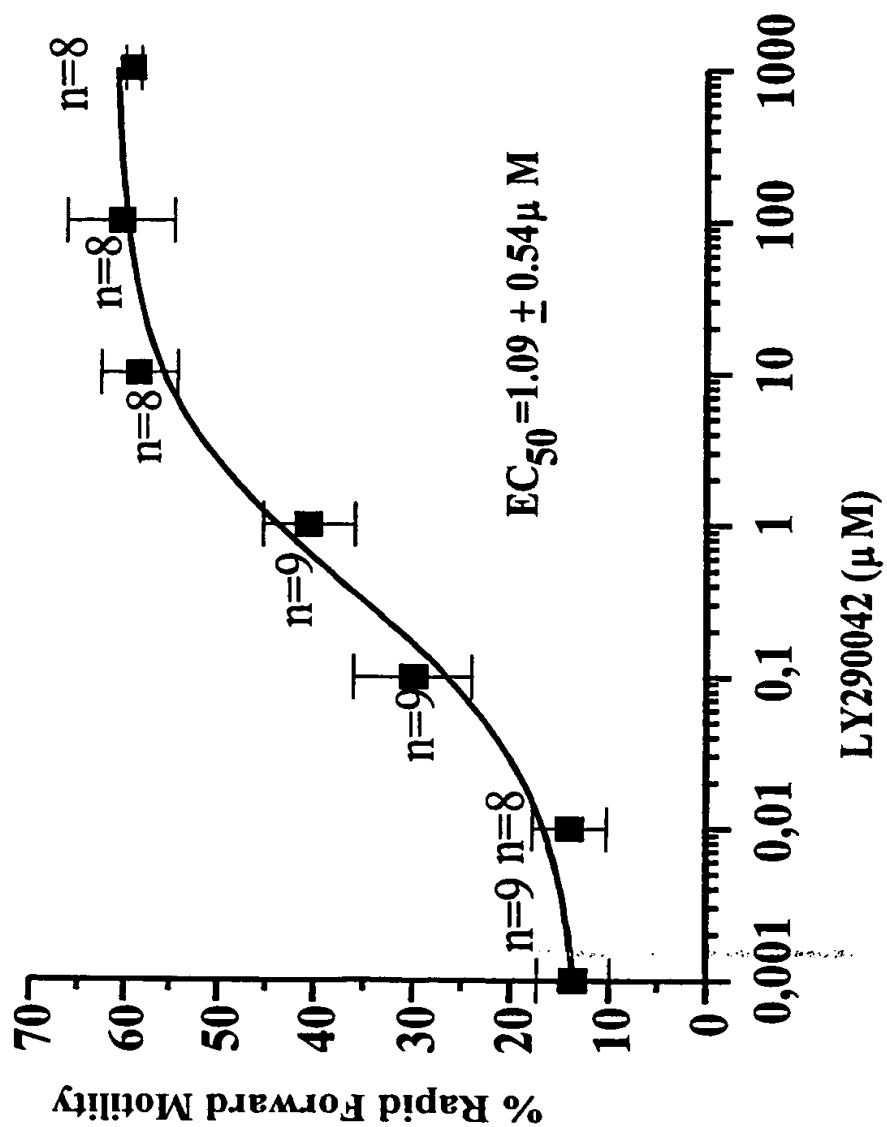


Fig. 5

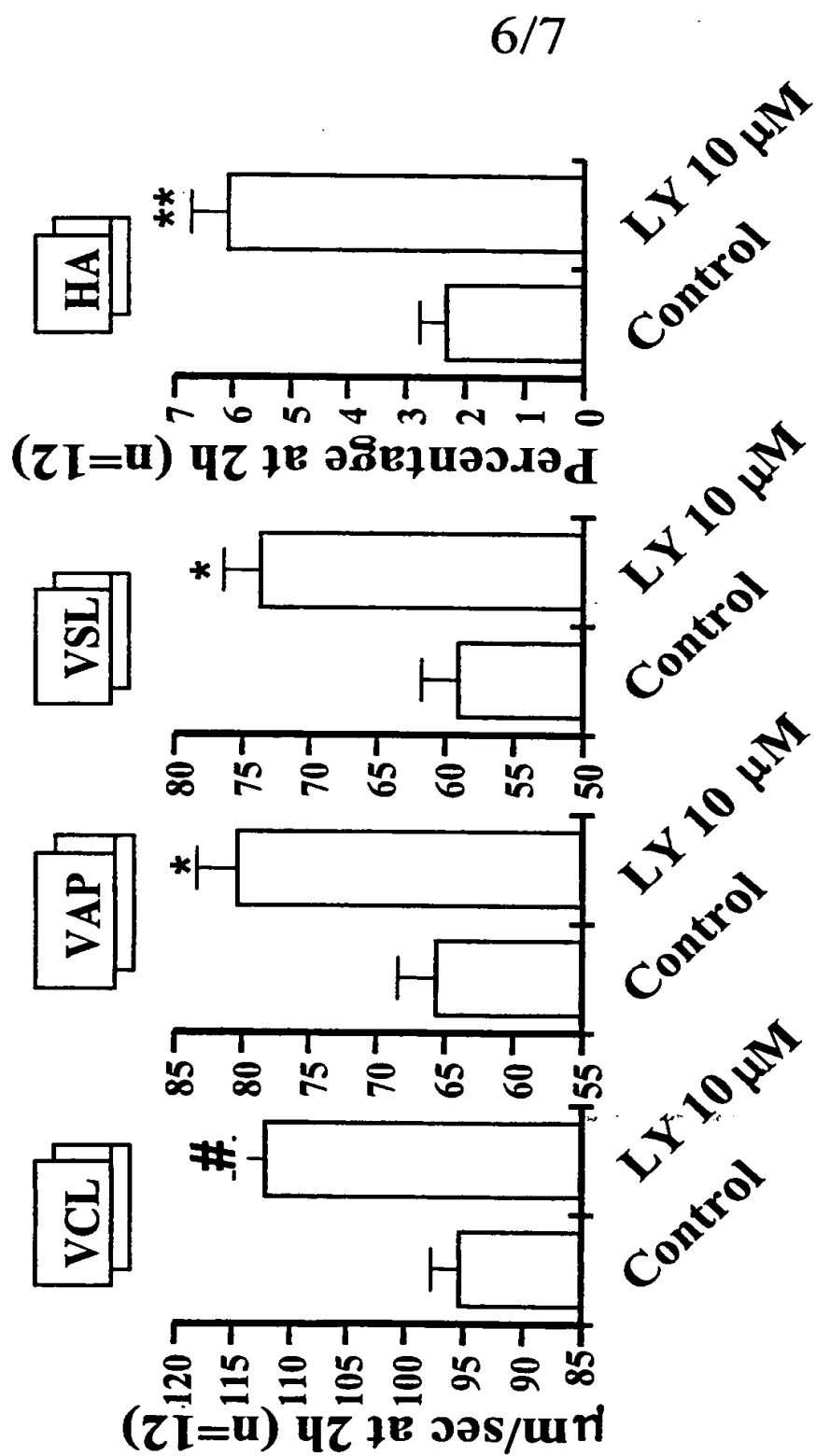


Fig. 6

7/7

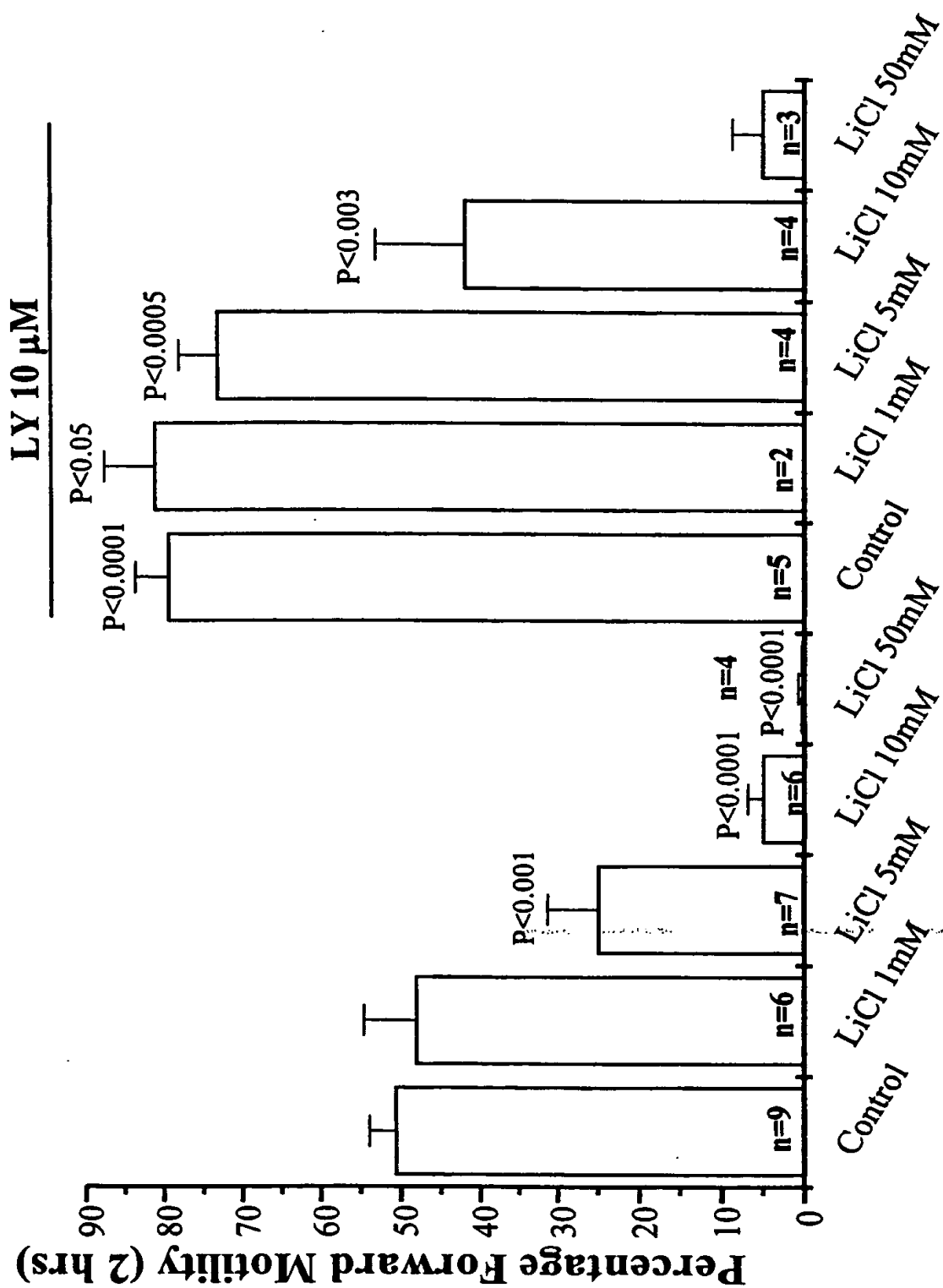


Fig. 7

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
1 February 2001 (01.02.2001)

PCT

(10) International Publication Number  
**WO 01/07021 A3**

(51) International Patent Classification<sup>7</sup>: **A61K 31/00**,  
31/352, 31/37, 31/5377, 35/52, A61D 19/00, 19/02, A01N  
1/02, A61P 15/08 // B01D 21/00, 43/00, B03B 7/00

(21) International Application Number: PCT/EP00/07108

(22) International Filing Date: 25 July 2000 (25.07.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
FI99A000171 26 July 1999 (26.07.1999) IT

(71) Applicant (for all designated States except US): **AP-  
PLIED RESEARCH SYSTEMS ARS HOLDING N.V.**  
[NL/NL]; Pietermaai 15, Curaçao (AN).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **LUCONI, Michaela**  
[IT/IT]; Via del Campo di Marte, 9, I-50137 Firenze (IT).  
**BALDI, Elisabetta** [IT/IT]; Via Ussi, 54, I-50018 Scan-  
dicci-Firenze (IT). **FORTI, Gianni** [IT/IT]; Via Lambrus-  
chini, 4/a, I-50134 Firenze (IT).

(74) Agent: **HASSA, Jürgen**; Serono International S.A., 12,  
chemin des Aulx, CH-1228 Plan-Les-Quates (CH).

(81) Designated States (national): AE, AG, AL, AM, AT, AU,  
AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE,  
DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU,  
ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS,  
LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ,  
PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT,  
TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States (regional): ARIPO patent (GH, GM,  
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian  
patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European  
patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE,  
IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG,  
CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:

— with international search report

(88) Date of publication of the international search report:  
6 December 2001

For two-letter codes and other abbreviations, refer to the "Guid-  
ance Notes on Codes and Abbreviations" appearing at the begin-  
ning of each regular issue of the PCT Gazette.

(54) Title: PROCESS FOR THE IMPROVEMENT OF SPERMATOZOA FERTILIZATION ACTIVITY

(57) Abstract: The invention relates to a process for the improvement of spermatozoa fertilization activity, in particular for the increase of spermatozoa motility by inhibiting the enzyme phosphatidylinositol-3 kinase (PI3K). The invention further relates to uses and methods of PI3K inhibitors in infertility and assisted reproduction techniques (ART) as well as to a medium for storage and/or transportation of spermatozoa comprising PI3K inhibitors. The PI3K inhibitors are selected from the group consisting of 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002), wortmannin, quercetin, and derivatives and analogues thereof.

WO 01/07021 A3

## PATENT COOPERATION TREATY

## PCT

## INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference <b>WO 0452</b>	<b>FOR FURTHER ACTION</b> see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No. <b>PCT/EP 00/ 07108</b>	International filing date (day/month/year) <b>25/07/2000</b>	(Earliest) Priority Date (day/month/year) <b>26/07/1999</b>
Applicant <b>APPLIED RESEARCH SYSTEMS ARS HOLDING N.V. et al.</b>		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 5 sheets.



It is also accompanied by a copy of each prior art document cited in this report.

## 1. Basis of the report

- a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.



the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

- b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :



contained in the international application in written form.



filed together with the international application in computer readable form.



furnished subsequently to this Authority in written form.



furnished subsequently to this Authority in computer readable form.



the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.



the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. ☐ **Certain claims were found unsearchable** (See Box I).

3. ☐ **Unity of invention is lacking** (see Box II).

4. With regard to the **title**,

the text is approved as submitted by the applicant.



the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

the text is approved as submitted by the applicant.



the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No.

as suggested by the applicant.



because the applicant failed to suggest a figure.



because this figure better characterizes the invention.



None of the figures.

## FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

## Continuation of Box I.2

Present claims 1-6,9-16,19-22,25 relate to products/compounds defined by reference to a desirable characteristic or property, namely their ability to inhibit PI-3 kinase .

The claims cover all products/compounds having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for only a very limited number of such products/compounds. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely on compounds of claim 7 and on the general idea underlying the present invention.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 00/07108

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K31/00 A61K31/352 A61K31/37 A61K31/557 A61K35/52  
 A61D19/00 A61D19/02 A01N1/02 A61P15/08 //B01D21/00,  
 B01D43/00, B03B7/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K A61D B01D B03B A01N C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

CHEM ABS Data, EPO-Internal

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>FRASER ET AL.: "Ca<sup>2+</sup>-regulating mechanisms that modulate bull sperm capacitation and acrosomal exocytosis as determined by Chlortetracycline analysis" MOL. REPROD. DEV., vol. 40, no. 2, 1995, pages 233-241, XP001009962</p> <p>page 233, right-hand column  page 234  page 237, right-hand column, paragraphs 3,4 -page 238, left-hand column  page 240, right-hand column, paragraph 1</p> <p>---  -/--</p>	<p>1-4,7,  13,14,  17,19,20</p>



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

## \* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- \*&\* document member of the same patent family

Date of the actual completion of the international search

29 June 2001

Date of mailing of the international search report

11/07/2001

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

Gac, G



## INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 00/07108

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NASS-ARDEN ET AL.: "Modulation of mammalian sperm motility by quercetin" MOL. REPROD. DEV., vol. 25, no. 4, April 1990 (1990-04), pages 369-373, XP001009971 the whole document	1-4,7
Y		15,16
A		21,23,25
X	ROLDAN ET AL.: "Is a Ca <sup>2+</sup> -ATPase involved in Ca <sup>2+</sup> regulation during capacitation and the acrosome reaction of guinea-pig spermatozoa ?" J. REPROD. FERTIL., vol. 85, no. 1, January 1989 (1989-01), pages 297-308, XP001009963 abstract page 304 see page 306 last sentence	1,4,9, 13,14, 19,20
X	HAMMERSTEDT ET AL.: "Motility, heat, and lactate production in ejaculated bovine sperm" ARCH. BIOCHEM. BIOPHYS., vol. 266, no. 1, October 1988 (1988-10), pages 111-123, XP001009970 see the whole document, especially Table I page 114	1-4,7,9, 10
Y		14-16
A		13
X	DASGUPTA ET AL.: "A possible role for Ca <sup>2+</sup> -ATPase in human sperm capacitation" J. REPROD. FERTIL., vol. 102, no. 1, September 1994 (1994-09), pages 107-116, XP001009960 page 108, right-hand column, paragraph 2 page 109, right-hand column, paragraphs 2,3 page 114, right-hand column, paragraph 1	1,2,6,7, 9,10, 15-17
A		4,13
X	BREITBART : "Calcium metabolism in spermatozoa: a target for contraception" ADV. CONTRACEPTIVE DELIVERY SYSTEMS, vol. 9, no. 2-3, 1993, pages 93-95, XP001009977 page 94; table I page 95, paragraph 1	1,2,7, 15-17
	---	
	--- -/--	

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 00/07108

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FRASER ET AL.: "Ca <sup>2+</sup> -related changes in mouse sperm capacitation state: a possible for Ca <sup>2+</sup> -ATPase" J. REPROD. FERTIL., vol. 96, no. 1, September 1992 (1992-09), pages 363-377, XP001009969 page 364 -page 365, paragraph 2	13,17
Y	page 370 -page 371 page 375, paragraph 2 page 376, paragraph 1 ---	14
X	FISHER ET AL.: "Phosphoinositide 3-kinase is involved in the induction of the human sperm acrosome reaction downstream of tyrosine phosphorylation" MOL. HUMAN REPROD., vol. 4, no. 9, September 1998 (1998-09), pages 849-855, XP001009974 the whole document	11,12
A	---	1,4-7,10
X	FENG ET AL.: "The c-kit receptor and its possible signaling transduction pathway in mouse spermatozoa" MOL. REPROD. DEV., vol. 49, no. 3, March 1998 (1998-03), pages 317-326, XP001009976 abstract page 318, left-hand column, paragraphs 2,3 page 319, right-hand column, paragraph 4 page 320, left-hand column, paragraph 1	11,12
A	---	1-5,7
P,X	BLUME-JENSEN ET AL.: "Kit/stem cell factor receptor-induced activation of phosphatidylinositol 3'-kinase is essential for male fertility" NATURE GENETICS, vol. 24, no. 2, February 2000 (2000-02), pages 157-162, XP001009975 page 158 page 160, right-hand column	11,12
Y	---	1,2
A	---	13,14
P,Y	JACK ET AL.: "Glycogen synthase kinase-3 phosphorylation- Evidence for a role for phosphoinositide-3-kinase signaling pathway in sperm motility regulation" BIOL. REPROD., vol. 62, no. suppl-1, 15 - 18 July 2000, pages 206-207, XP001009990 see abstract 252 --- -/--	1,2,15, 16

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 00/07108

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
E	EP 1 057 505 A (MG PHARMACY LTD) 6 December 2000 (2000-12-06) column 2, line 48 - line 55 column 3, line 38 column 4, line 30 - line 58 ----	21-23, 25
A	ARAVINDAKSHAN ET AL.: "Studies on germinal effects of quercetin, a naturally occurring flavonoid" MUTAT. RES., vol. 144, 1985, pages 99-106, XP001009965 the whole document ----	13-17
A	WO 95 29673 A (JUNE C.) 9 November 1995 (1995-11-09) the whole document ----	1, 7
A	DATABASE WPI Week 199620 Derwent Publications Ltd., London, GB; AN 1996-195278 XP002170825 & JP 08 066183 A (SHINGIJUTSU JIGYODAN), 12 March 1996 (1996-03-12) abstract ----	21-23, 25
A	DATABASE WPI Week 199303 Derwent Publications Ltd., London, GB; AN 1993-024773 XP002170826 & KR 9 202 285 B (PACIFIC CHEM. CO.), 21 March 1992 (1992-03-21) abstract ----	21-23, 25
A	US 5 733 759 A (LOVERINE ET AL.) 31 March 1998 (1998-03-31) the whole document ----	1, 2, 7
A	US 4 774 088 A (SHOBHANA V.) 27 September 1988 (1988-09-27) the whole document -----	21-23, 25

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 00/07108

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
EP 1057505	A	06-12-2000	JP 2000345724 A	12-12-2000
WO 9529673	A	09-11-1995	AU 706761 B	24-06-1999
			AU 2398295 A	29-11-1995
			AU 5013499 A	16-12-1999
			CA 2189055 A	09-11-1995
			EP 0758232 A	19-02-1997
			JP 9512553 T	16-12-1997
JP 08066183	A	12-03-1996	JP 2991620 B	20-12-1999
KR 9202285	B	21-03-1992	NONE	
US 5733759	A	31-03-1998	AU 668548 B	09-05-1996
			AU 3786493 A	05-10-1993
			BG 62891 B	31-10-2000
			BG 99033 A	29-09-1995
			BR 9306047 A	18-11-1997
			CA 2131704 A	16-09-1993
			EP 0630403 A	28-12-1994
			HU 69991 A	28-09-1995
			JP 7506963 T	03-08-1995
			NZ 251069 A	26-01-1996
			RO 116095 B	30-10-2000
			WO 9318142 A	16-09-1993
US 4774088	A	27-09-1988	AU 6897687 A	28-07-1987
			EP 0258290 A	09-03-1988
			WO 8704072 A	16-07-1987

# PATENT COOPERATION TREATY

## PCT

### INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 452 WO		<b>FOR FURTHER ACTION</b> See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. PCT/EP00/07108	International filing date (day/month/year) 25/07/2000	Priority date (day/month/year) 26/07/1999	
International Patent Classification (IPC) or national classification and IPC A61K31/00			
Applicant APPLIED RESEARCH SYSTEMS ARS HOLDING N.V. et al.			



1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.
2. This REPORT consists of a total of 7 sheets, including this cover sheet.

☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☒ Priority
- III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☐ Certain observations on the international application

Date of submission of the demand 19/01/2001	Date of completion of this report 12.10.2001
Name and mailing address of the international preliminary examining authority:  European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Langer, A Telephone No. +49 89 2399 7809 

PCT Rec'd 28 JAN 2002

**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT**

International application No. PCT/EP00/07108

**I. Basis of the report**

1. With regard to the **elements** of the international application (*Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17)*):

**Description, pages:**

1-16 as originally filed

**Claims, No.:**

1-25 as originally filed

**Drawings, sheets:**

1/7-7/7 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:

**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT**

International application No. PCT/EP00/07108

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

*(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)*

6. Additional observations, if necessary:

**II. Priority**

1. ☐ This report has been established as if no priority had been claimed due to the failure to furnish within the prescribed time limit the requested:

☐ copy of the earlier application whose priority has been claimed.

☐ translation of the earlier application whose priority has been claimed.

2. ☐ This report has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid.

Thus for the purposes of this report, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:  
**see separate sheet**

**III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability**

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application.

☒ claims Nos. 1-6, 9-16, 19-22, 25 (partially) as to N, IS, IA; 1-10, 13, 14, 17, 19, 20 as to IA.

because:

☒ the said international application, or the said claims Nos. 1-10, 13, 14, 17, 19, 20 as to IA relate to the following subject matter which does not require an international preliminary examination (*specify*):  
**see separate sheet**

☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion

# INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/EP00/07108

could be formed.

☒ no international search report has been established for the said claims Nos. 1-6, 9-16, 19-22, 25 (partially).

2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

☐ the written form has not been furnished or does not comply with the standard.

☐ the computer readable form has not been furnished or does not comply with the standard.

## V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

### 1. Statement

Novelty (N)	Yes:	Claims 8, 18, 24 → 24234002
	No:	Claims 1-6, 9-16, 19-22, 25 (partially), 7, 17, 23
Inventive step (IS)	Yes:	Claims 8, 18, 24
	No:	Claims 1-6, 9-16, 19-22, 25 (partially), 7, 17, 23
Industrial applicability (IA)	Yes:	Claims 11, 12, 15, 16, 18, 21-25
	No:	Claims

2. Citations and explanations  
see separate sheet



**Re Item II**

**Priority**

The priority claimed for the present application is valid for claims 1-6, 8, 11, 12, partially valid for claims 7, 9, 10, 15-18 and not valid for claims 13, 14, 19-25. The document cited as P-documents in the international search report are therefore considered as prior art in terms of Rule 64.1 (a) PCT for claims 7, 9, 10, 15-18 (partially) and for claims 13, 14, 19-25.

**Re Item III**

**Non-establishment of opinion with regard to novelty, inventive step and industrial applicability**

**Claims 1-6, 9-16, 19-22, 25** relate to products/compounds defined by reference to a desirable characteristic or property, namely their ability to inhibit PI-3 kinase.

The claims cover all products/compounds having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within in the meaning of Article 5 PCT for only a very limited number of such products/compounds. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely on compounds of claim 7 and on the general idea underlying the present invention.

Claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (rule 66.1(e) PCT). The following examination has therefore only been carried out for claims 7, 8, 17, 18, 23, 24 as well as claims 1-6, 9-16, 19-22, 25 (partially, i.e. limited to the searched matter).

**Claims 1-10, 13, 14, 17, 19, 20** relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(i) PCT).

**Re Item V**

Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Reference is made to the following documents. If not indicated otherwise, the relevant passages are those cited in the international search report.

D1: FRASER ET AL.: 'Ca<sup>2+</sup>-regulating mechanisms that modulate bull sperm capacitation and acrosomal exocytosis as determined by Chlortetracycline analysis' MOL. REPROD. DEV., vol. 40, no. 2, 1995, pages 233-241, XP001009962

D2: NASS-ARDEN ET AL.: 'Modulation of mammalian sperm motility by quercetin' MOL. REPROD. DEV., vol. 25, no. 4, April 1990 (1990-04), pages 369-373, XP001009971

additional relevant passages: p. 370

D3: ROLDAN ET AL.: 'Is a Ca<sup>2+</sup>-ATPase involved in Ca<sup>2+</sup> regulation during capacitation and the acrosome reaction of guinea-pig spermatozoa?' J. REPROD. FERTIL., vol. 85, no. 1, January 1989 (1989-01), pages 297-308, XP001009963

additional relevant passages: Fig. 1, Table 1

D4: HAMMERSTEDT ET AL.: 'Motility, heat, and lactate production in ejaculated bovine sperm' ARCH. BIOCHEM. BIOPHYS., vol. 266, no. 1, October 1988 (1988-10), pages 111-123, XP001009970

additional relevant passages: p. 112, right column - p. 113, left column

D5: DASGUPTA ET AL.: 'A possible role for Ca<sup>2+</sup>-ATPase in human sperm capacitation' J. REPROD. FERTIL., vol. 102, no. 1, September 1994 (1994-09), pages 107-116, XP001009960

additional relevant passages: p. 109, 1st paragraph

D6: BREITBART : 'Calcium metabolism in spermatozoa: a target for contraception' ADV. CONTRACEPTIVE DELIVERY SYSTEMS, vol. 9, no. 2-3, 1993, pages 93-95, XP001009977

D7: FRASER ET AL.: 'Ca<sup>2+</sup>-related changes in mouse sperm capacitation state: a possible role for Ca<sup>2+</sup>-ATPase' J. REPROD. FERTIL., vol. 96, no. 1, September 1992 (1992-09), pages 363-377, XP001009969

D8: FISHER ET AL.: 'Phosphoinositide 3-kinase is involved in the induction of

the human sperm acrosome reaction downstream of tyrosine phosphorylation' MOL. HUMAN REPROD., vol. 4, no. 9, September 1998 (1998-09), pages 849-855, XP001009974

additional relevant passages: p. 850, right column

D9: FENG ET AL.: 'The c-kit receptor and its possible signaling transduction pathway in mouse spermatozoa' MOL. REPROD. DEV., vol. 49, no. 3, March 1998 (1998-03), pages 317-326, XP001009976

## **2. Novelty (Art. 33 (2) PCT) and Inventive Step (Art. 33 (3) PCT)**

**Claims 1, 2, 6, 7, 11, 12, 15-17, 21-23 and 25** lack novelty over documents D1-D9 in terms of Art. 33 (2) PCT. The novelty of **claims 3, 4** is destroyed by documents D1-D5, D7-D9. **Claim 5** lacks novelty over documents D8 and D9, **claim 9** over documents D1-D3, D5-D8, **claim 10** over documents D1-D3, D5, D7-D9, while document D3 destroys the novelty of **claims 13, 14, 19 and 20**.

The substance LY294002 as well as its inhibitory effect on P13K is not disclosed nor indicated by the prior art. **Claims 8, 18 and 24** are therefore novel and inventive in terms of Art. 33 (2) (3) PCT.

## **3. Industrial Applicability (Art. 33 (4) PCT)**

For the assessment of the present **claims 1-10, 13-20** on the question whether they are industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.